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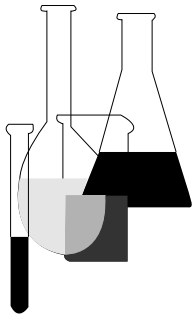
Ecological Effects Test Guidelines

OPPTS 850.5400
Algal Toxicity, Tiers I and II



“Public Draft”

INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

Public Draft Access Information: This draft guideline is part of a series of related harmonized guidelines that need to be considered as a unit. *For copies:* These guidelines are available electronically from the EPA Public Access Gopher (gopher.epa.gov) under the heading ‘‘Environmental Test Methods and Guidelines’’ or in paper by contacting the OPP Public Docket at (703) 305–5805 or by e-mail: guidelines@epamail.epa.gov.

To Submit Comments: Interested persons are invited to submit comments. By mail: Public Docket and Freedom of Information Section, Office of Pesticide Programs, Field Operations Division (7506C), Environmental Protection Agency, 401 M St. SW., Washington, DC 20460. In person: bring to: Rm. 1132, Crystal Mall #2, 1921 Jefferson Davis Highway, Arlington, VA. Comments may also be submitted electronically by sending electronic mail (e-mail) to: guidelines@epamail.epa.gov.

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OPPTS 850.5400 Algal toxicity, Tiers I and II.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background.** The source material used in developing this harmonized OPPTS test guideline are 40 CFR 797.1050 Algal Acute Toxicity Test; OPP 123–2 Growth and Reproduction of Aquatic Plants (Tier 2) (Pesticide Assessment Guidelines, Subdivision J—Hazard Evaluation; Nontarget Plants) EPA report 540/09-82-020, 1982; and OECD 201, Algal Growth Inhibition Test.

(b) **Purpose.** This guideline is intended for use in developing data on the acute toxicity of chemical substances and mixtures (chemicals) subject to environmental effects test regulations, and was written specifically for *Selenastrum capricornutum* and *Skeletonema costatum* (see paragraph (d)(2)(iii) of this guideline). Use of *Anabaena flos-aquae* or *Navicula pelliculosa* may require some specific modifications in test procedures. This guideline prescribes test procedures and conditions using freshwater and marine algae to develop data on the phytotoxicity of chemicals. EPA will use data from these tests in assessing the hazard of a chemical to the environment.

(c) **Definitions.** The definitions in section 3 of the Toxic Substances Control Act (TSCA) and the definitions in 40 CFR Part 792—Good Laboratory Practice Standards apply to this test guideline. The following definitions also apply to this guideline:

Algicidal means having the property of killing algae.

Algistatic means having the property of inhibiting algal growth.

ECX means the experimentally derived chemical concentration that is calculated to effect X percent of the test criterion.

Growth means an increase in an algal population based on the number and/or weight of algal cells per volume of nutrient medium or test solution in a specified period of time.

Static system means for this test a system in which old nutrient medium (test solution) is not renewed or replaced during the period of the test.

(d) **Test procedures—**(1) **Summary of the test.** (i) In preparation for the test, fill test containers with appropriate volumes of nutrient medium and/or test solution. Start the test by introducing algae into the test and control containers in the growth chambers. Environmental conditions within the growth chambers are established at predetermined limits.

(ii) At the end of 96 h, and at the end of 24, 48, and 72 h if possible, enumerate the algal cells in all containers to determine inhibition or stimulation of growth and the pattern of growth in test containers compared to controls. Use data to define the concentration-response curve, and calculate the EC50 value at these times.

(2) **Range-finding test.** (i) A range-finding test should be conducted to determine if definitive testing is necessary, and if so, test chemical concentrations for the definitive test. Water solubility of the test chemical (as well as other physical chemical characteristics, e.g. volatility) should be determined before definitive testing. A validated analytical method should also be developed prior to any toxicity testing.

(ii) Algae are exposed to a widely spaced (e.g. log interval) chemical concentration series. The lowest value in the series, exclusive of controls, should be at the chemical's detection limit. The upper value, for water soluble compounds, should be the saturation concentration. A minimum of three replicates is required: Nominal concentrations of the chemical are acceptable if it is determined that definitive testing is not required.

(iii) The test is performed once for each of the recommended algal species or selected alternates. Test chambers should contain equal volumes of test solution and approximately 1×10^4 *S. capricornutum* (misidentified previously as as *Monoraphidium capricornutum*, correctly also known as *Raphidocelis subcapitata* Korsikov), *N. pelliculosa*, or *A. flos-aquae* cells per milliliter or 7.7×10^4 *S. costatum* cells per milliliter of test solution. The algae should be exposed to each concentration of test chemical for up to 96 h.

(iv) Definitive testing may not be necessary if the highest chemical concentration tested (water saturation concentration or 1,000 mg/L) results in less than a 50 percent reduction in growth. However, if the lowest concentration tested (analytical detection limit) results in greater than a 50 percent reduction in growth definitive testing is necessary. If testing a pesticide under FIFRA at the maximal labeled dosage, a minimum of three replicates for each test chemical concentration is required.

(3) **Definitive test.** (i) The purpose of the definitive test is to determine the concentration response curves, the EC50's for algal growth for each species tested, with a minimum amount of testing beyond the range-finding test.

(ii) Algae should be exposed to five or more concentrations of the test chemical in a geometric series in which the ratio is between 1.5 and 2.0 (e.g. 2, 4, 8, 16, 32, and 64 mg/L). Often it is possible to choose test chemical concentrations based on the anticipated slope of the concentration-response curve, and these concentrations should bracket the expected test end-points. Algae are to be placed in a minimum of three replicate test containers for each concentration of test chemical and control.

With the exception of the use of four replicates for *N. pelliculosa*, more than three replicates may be required to provide sufficient data. Each test chamber should contain equal volumes of test solution and approximately 1×10^4 *Selenastrum, Navicula*, or *Anabaena* cells per milliliter or 7.7×10^4 *Skeletonema* cells per milliliter of test solution. The chemical concentrations should result in greater than 90 percent of algal growth being inhibited or stimulated at the highest concentrations of test substance compared to controls or that the test concentrations should bracket the expected EC50 value.

(iii) Every test is to include a control (negative control) consisting of the same nutrient medium, conditions, procedures, and algae from the same culture, except that none of the test substance is added. If a carrier is present in any of the test chambers, a separate carrier control is required.

(iv) Positive controls using zinc chloride as a reference chemical should also be run periodically. The purpose of a positive control with a reference chemical is to determine that the test algae are responding to a known chemical in the expected manner. If the algae are responding to subsequent reference chemical tests consistently, it is assumed that the algae will respond to other chemicals consistently. Changes in algal response caused by such factors as poor nutrition, genetic drift, and contaminants may not be detected by negative controls, yet may still influence test results. At least three concentrations of the reference chemical are run at or near the expected median effect level.

(v) The test begins when algae (inocula) from 3– to 7–day–old stock cultures are placed in the test chambers containing test solutions having the appropriate concentrations of the test substance. The mean cell volume of inocula should be approximately 35–45 $\mu g/m^3$ at the onset of testing. Algal growth in controls should reach the logarithmic growth phase by 96 h (at which time the number of algal cells should be approximately 1.5×10^6/mL for *Skeletonema* or 3.5×10^6/mL for *Selenastrum*. If logarithmic growth cannot be demonstrated, the test is to be repeated. At the end of 96 h, and, if possible, at the end of 24, 48, and 72 h, the algal growth response (number or weight of algal cells per milliliter) in all test containers and controls is to be determined by an indirect (spectrophotometry, electronic cell counters, dry weight, etc.) or a direct (actual microscopic cell count of at least 400 cells per flask) method. Indirect methods are to be calibrated by a direct microscopic count or data should be presented that relate electronic counts with microscopic counts. The percentage inhibition or stimulation of growth for each concentration, EC50, and the concentration-response curves are determined from these counts.

(vi) A particle counter or microscopic counting cannot be used for *Anabaena* unless the filaments are broken up and dispersed using a syringe, ultrasonic bath, or blender. Limited use of sonification is allowed

for *Anabaena*. The procedure used to break up the filaments should result in consistent filament lengths across treatments and replicates. Sonification, ultrasonic bath, blender, syringe, or any other methods of cell separation, other than manual or rotary shaking are not allowed for *Selenastrum*, *Skeletonema*, or *Navicula*.

(vii) At the end of the definitive test, the following additional analyses of algal growth response are to be performed:

(A) Determine whether the altered growth response between controls and test algae (in highest test chemical concentrations) was due to a change in relative cell numbers, cell sizes, or both. Also note any unusual cell shapes, color differences, differences in chloroplast morphology, flocculations, adherence of algae to test containers, or aggregation of algal cells. These observations are qualitative and descriptive, and are not used in end-point calculations. They can be useful in determining additional effects of tested chemicals.

(B) In test concentrations where growth is maximally inhibited, algistatic effects may be differentiated from algicidal effects by the following two methods.

(*1*) Add 0.5 mL of a 0.1 percent solution (weight/volume) of Evans blue stain to a 1–mL aliquot of algal suspension from a control container and to a 1–mL aliquot of algae from the test container having the lowest concentration of test chemical which completely inhibited algal growth (if algal growth was not completely inhibited, select an aliquot of algae for staining from the test container having the highest concentration of test chemical which inhibited algal growth). Wait 10 to 30 min, examine microscopically, and determine the percent of the cells which stain blue (indicating cell mortality). A staining control is to be performed concurrently using heat-killed or formaldehyde-preserved algal cells; 100 percent of these cells should stain blue.This method will work for *Skeletonema* and possibly *Navicula*, but probably will not work with *Selenastrum* or *Anabaena*.

(*2*) Remove 0.5 mL aliquots of test solution containing growth-inhibited algae from each replicate test container having the concentration of test substance evaluated in paragraph (d)(3)(vii)(B)(*1*) of this guideline. Combine these aliquots into a new test container and add a sufficient volume of fresh nutrient medium to dilute the test chemical to a concentration which does not affect growth. Incubate this subculture under the environmental conditions used in the definitive test for a period of up to 9 days, and observe periodically (e.g. every other day) for algal growth (direct or indirect methods) to determine if the algistatic effect noted after the 96–h test is reversible. This subculture test may be discontinued as soon as growth occurs.

(4) **Analytical measurements**—(i) **Chemical.** (A) Water of sufficient quality (e.g. ASTM Type I water) is to be used in the preparation of the nutrient medium. The pH of the test solution and controls is to be measured at the beginning and at the end of the definitive test. The concentration of test chemical in the test containers is to be determined at the beginning and end of the definitive test by standard analytical methods which have been validated prior to the test. An analytical method is unacceptable if likely degradation products of the chemical, such as hydrolysis and oxidation products, give positive or negative interference to the method. To be acceptable, the analytical method must be corrected for these interferences.

(B) At the end of the test and after aliquots have been removed for algal growth-response determinations, microscopic examination, mortal staining, or subculturing, the replicate test containers for each chemical concentration may be pooled into one sample. An aliquot of the pooled sample may then be taken and the concentration of test chemical determined after all algal cells have been removed. In addition, the concentration of test chemical associated with the algae alone may be determined. Separate and concentrate the algal cells from the test solution by centrifuging or filtering the remaining pooled sample and measure the test substance concentration in the algal-cell concentrate.

(ii) **Numerical.** Algal growth response (as percent of inhibition or stimulation in the test solutions compared to the controls) is calculated at the end of the test. Mean and standard deviation should be calculated and plotted for each treatment and control. Appropriate statistical analyses (see paragraphs (g)(1) and (g)(2) of this guideline) should provide a goodness-of-fit determination for the concentration response curves. The concentration response curves are plotted using the mean measured test solution concentrations obtained in the test chambers at the end of the test. Results from the recovery phase are used to determine the algistatic concentration (refer to paragraph (g)(3) of this guideline). Various statistical procedures for modeling continuous toxicity data are available and can be used (see paragraph (g)(4) of this guideline).

(e) **Test conditions**—(1) **Test species.** Species of algae recommended as test organisms for this test are the freshwater green alga, *S. capricornutum* (or *R. subcapitata*), the marine diatom, *S. costatum*, the freshwater diatom, *N. pelliculosa*, and the blue-green alga or cyanobacterium, *A. flos-aquae*. Algae to be used in acute toxicity tests may be initially obtained from commercial sources and subsequently cultured using sterile technique. Toxicity testing should not be performed until algal cultures are shown to be actively growing (i.e. capable of logarithmic growth within the test period) in at least two subcultures lasting 7 days each prior to the start of the definitive test. All algae used for a particular test should be from the same source and the same stock cul-

ture. Also, the clone of all species should be specified. Test algae should not have been used in a previous test, either in a treatment or a control.

(2) **Facilities**—(i) **General.** (A) Facilities needed to perform this test include: A growth chamber or a controlled environment room that can hold the test containers and will maintain the air temperature, lighting intensity, and photoperiod specified in this test guideline, apparatus for culturing and enumerating algae, a source of water of acceptable quality, and apparatus for carrying out analyses of the test chemical.

(B) Disposal facilities should be adequate to accommodate spent glassware, algae, and test solutions at the end of the test, and any bench covering, lab clothing, or other contaminated materials.

(ii) **Test containers.** Erlenmeyer flasks should be used for test containers. The flasks may be of any volume between 125 and 500 mL as long as the same size is used throughout a test and the test solution volume does not exceed 50 percent of the flask volume.

(iii) **Cleaning and sterilization.** New test containers may contain substances which inhibit growth of algae. They are therefore to be cleaned thoroughly and used several times to culture algae before being used in toxicity testing. All glassware used in algal culturing or testing is to be cleaned and sterilized prior to use according to standard good laboratory practices. These practices include rinsing in a solvent followed by rinsing with acid.

(iv) **Conditioning.** Test containers should be conditioned, if needed, by a rinse with the appropriate test solutions prior to the start of the test. Decant and add fresh test solutions after an appropriate conditioning period for the test chemical.

(v) **Nutrient medium.** (A) Formulation and sterilization of nutrient medium used for algal culture and preparation of test solutions should conform to those currently recommended by the EPA for freshwater and marine algal bioassays under paragraph (g)(1) of this guideline. Chelating agents are included in the nutrient medium for optimum cell growth. No chelating agents are to be included in the nutrient medium used for test solution preparation if it is suspected that the chelater will interact with the test chemical. For some chemicals, the stock solution must be prepared with dilution water (e.g. ASTM Type I water). Volumetric addition should be limited to prevent dilution of the nutrients. Nutrient medium should be freshly prepared for algal testing and may be dispensed in appropriate volumes in test containers and sterilized by autoclaving or filtering by using a clean water source. The pH of the nutrient medium is to be $7.5 (\pm 0.1)$ for *Selenastrum*, $8.1 (\pm 0.1)$ for *Skeletonema*, $7.5 (\pm 0.1)$ for *Navicula*, and $7.5 (\pm 0.1)$ for *Anabaena* at the start of the test and may be adjusted prior to test chemical addition with 0.1N or 1N NaOH or HC1.

(B) Dilution water used for preparation of nutrient medium and test solutions should be of sufficient quality (e.g. ASTM Type I water). Saltwater for marine algal nutrient medium and test solutions should be prepared by adding a commercial synthetic sea salt formulation or a modified synthetic seawater formulation to distilled/deionized water to a concentration of 30 ppt (24 to 35 g/kg).

(vi) **Carriers.** Nutrient medium is to be used in making stock solutions of the test chemical. If a carrier (or solvent) other than nutrient medium is absolutely necessary to dissolve the chemical, the volume used should not exceed the minimum volume necessary to dissolve or suspend the chemical in the test solution. The upper limit of carrier volume is 0.5 mL/L and the same amount of carrier should be added to each concentration (refer to paragraph (g)(1) of this guideline).

(3) **Test parameters.** (i) The test temperature is to be 24 °C for *Selenastrum*, *Navicula*, and *Anabaena*, and 20 °C for *Skeletonema*. Excursions from the test temperature should be no greater than ± 2 °C. Temperature should be recorded hourly during the test. A continuous recording device is suitable for this purpose.

(ii) Test chambers containing *Selenastrum*, *Navicula*, and *Anabaena*, must be illuminated continuously; those containing *Skeletonema* are to be provided a 14–h light/10–h dark photoperiod. Fluorescent lights providing 4.3 K lx (4,306 lm/m² or 400 ± 10% fc) for *Selenastrum*, *Skeletonema*, and *Navicula*, and 2.2 K lx for *Anabaena* are to be used. These lamps should have a photosynthetically active radiation of approximately 66.5 ± 10% μEin/m²/sec. Light intensities (or light fluence rate) should be measured at each test chamber position at the approximate level of the test solution.

(iii) Stock algal cultures should be shaken on a rotary shaking apparatus. Test containers also should be placed on a rotary shaking apparatus and oscillated at approximately 100 cycles/min for *Selenastrum* and at approximately 60 cycles/min for *Skeletonema* during the test. If clumping of *Skeletonema* is experienced or anticipated, hand shaking once or twice a day is acceptable. The rate of oscillation should be determined at the beginning of the test or at least once daily during testing if the shaking rate is changed or changes.

(iv) The pH of nutrient medium in which algae are subcultured is to be 7.5 (± 0.1) for *Selenastrum*, 8.1 (± 0.1) for *Skeletonema*, 7.5 (± 0.1) for *Navicula*, and 7.5 (± 0.1) for *Anabaena*, and is not adjusted after the addition of the algae. The pH of all test solutions is to be measured at the beginning and end of the test. If the test chemical is highly acidic and reduces the pH of the test solution below 5.0 at the first measurement, appropriate adjustments to pH should be considered, and the test solution measured for pH on each day of the test.

(v) Light intensity should be monitored at the beginning of the test at the level of the test solution or at each test chamber position. If it is suspected that light intensity has changed, monitoring more often during the test will be necessary.

(f) **Reporting.** The sponsor must submit to the EPA all data developed by the test including those that are suggestive or predictive of acute phytotoxicity. In addition to the reporting requirements as specified under EPA Good Laboratory Practice Standards, 40 CFR part 792, subpart J, the following specific information is to be reported:

(1) Detailed information about the test organisms, including the scientific name, method of verification, strain, and source.

(2) Control charts of growth in the nontreatment and solvent controls for each toxicity test.

(3) A description of the test chambers and containers, the volumes of solution in the containers, the way the test was begun (e.g. conditioning, test substance additions, etc.), the number of replicates, the temperature, the lighting, and method of incubation, oscillation rates, and type of apparatus. Specific modifications in test procedures due to using *Anabaena* or *Navicula* must be noted.

(4) The concentration of the test chemical in the control and in each treatment at the end of the test and the pH of the solutions.

(5) The number of algal cells per milliliter in each treatment and control and the method used to derive these values at the beginning, at 24, 48, and 72 h, and at the end of the test; the percentage of inhibition or stimulation of growth relative to controls; and other adverse effect in the control and in each treatment.

(6) The 96–h EC50 values, and when sufficient data have been generated, the 24–, 48–, and 72–h EC50s and 95 percent confidence limits, the methods used to derive these values, the data used to define the shape of the concentration-response curve and the goodness-of-fit determination. Electronic data submission (raw data) is encouraged to reduce data entry time required to conduct statistical analyses.

(7) Methods and data records of all chemical analyses and test substance concentrations, including method validations and reagent blanks.

(8) The results of analyses such as: Microscopic appearance of algae, size or color changes, percent mortality of cells and the fate of subcultured cells, the concentration of test substance associated with algae and test solution supernate or filtrate.

(9) If the range-finding test showed that the highest concentration of the chemical tested (not less than 1,000 mg/L or saturation concentration)

had no effect on the algae, report the results and concentration and a statement that the chemical is of minimum phytotoxic concern.

(10) If the range-finding test showed greater than a 50 percent inhibition of algal growth at a test concentration at or below the analytical detection limit, report the results, concentration, and a statement that the chemical is phytotoxic at or below the analytical detection limit.

(g) **References.** The following references should be consulted for additional background material on this test guideline.

(1) American Society for Testing and Materials. ASTM E1218–20. Standard guide for conducting 96–h toxicity tests with microalgae. In: 1991 Annual Book of ASTM Standards, Vol. 11.04: Pesticides; resource recovery; hazardous substances and oil spill response; waste disposal; biological effects. pp 845–856 (1991).

(2) American Society for Testing and Materials. ASTM D 3978–80. Standard practice for algal growth potential testing with *Selenastrum capricornutum*. In: 1991 Annual Book of ASTM Standards, Vol. 11.04: Pesticides; resource recovery; hazardous substances and oil spill response; waste disposal; biological effects. pp 32–36 (1991).

(3) Payne, A.G. and R.H. Hall. A method for measuring algal toxicity and its application to the safety assessment of new chemicals. pp 171-180 in L.L. Marking and R.A. Kimerle (eds.). Aquatic Toxicology, ASTM STM 667, American Society for Testing and Materials, Philadelphia, PA (1979).

(4) Bruce, R.D. and D.J. Versteeg. A statistical procedure for modeling continuous toxicity data. *Environmental Toxicology and Chemistry* 11:1485-1494 (1992).